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Communication

Identification and Characterization of a Novel Cytokine, THANK, a TNF Homologue That Activates Apoptosis, Nuclear Factor-κB, and c-Jun NH_2-Terminal Kinase*

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By using the amino acid sequence motif of tumor necrosis factor (TNF), we searched the expressed sequence tag data base and identified a novel full-length cDNA encoding 285 amino acid residues and named it THANK. THANK is a type II transmembrane protein with 15–20% overall amino acid sequence homology to TNF, LT-α, FasL, and LIGHT, all members of the TNF family. The mRNA for THANK was expressed at high levels by peripheral blood leukocytes, lymph node, spleen, and thymus and at low levels by small intestine, pancreas, placenta, and lungs. THANK was also prominently expressed in hematopoietic cell lines. The recombinant purified protein expressed in the baculovirus system had an approximate molecular size 20 kDa with amino-terminal sequence of AVQGP. Treatment of human myeloid U937 cells with purified THANK activated nuclear transcription factor-κB (NF-κB) consisting of p50 and p65. Activation was time- and dose-dependent, beginning with as little as a 1 pM amount of the cytokines and as early as 15 min. Under the same conditions, THANK also activated c-jun NH_2-terminal kinase (JNK) in U937 cells. THANK also strongly suppressed the growth of tumor cell lines and activated caspase-3. Although THANK had all the activities and potency of TNF, it did not bind to the TNF receptors. Thus our results indicate that THANK is a novel cytokine that belongs to the TNF family and activates apoptosis, NF-κB, and JNK through a distinct receptor.

In 1984, we reported the isolation of two homologous cytokines that can inhibit the growth specifically of tumor cells (1–7) and named them TNF-α[1] and TNF-β (also called lymphotoxin). Since then over 15 members of this family have been identified, including FasL, CD27L, CD30L, CD40L, OX-40L, 4–1BBL, LT-β, TWEAK, TRAIL, RANKL/TRANCE, LIGHT, VEGI, and APRIL (8–16). At the amino acid sequence level, various members of the TNF family are 20–25% homologous to each other. Most members of this family play an important role in gene activation, proliferation, differentiation, and apoptosis. These ligands interact with the corresponding receptor, also members of the TNF receptor family, and activate the transcription factors NF-κB and AP-1 (9, 17), a stress-activated protein kinase (c-jun NH_2-terminal protein kinase, JNK), and a cascade of caspases.

By searching an expressed sequence tag (EST) data base using the amino acid sequence motif of TNF, we identified a novel member of the TNF family, named THANK, for TNF homologue that activates apoptosis, NF-κB, and JNK. We found that this cytokine was primarily expressed by hematopoietic cells. The recombinant THANK activated NF-κB, c-jun NH_2-terminal kinase, caspase-3, and displayed antiproliferative effects in U937 cells through binding sites distinct from those for TNF.

MATERIALS AND METHODS

Identification, Cloning, Expression, and Purification of THANK—Using high throughput automated DNA sequence analysis of randomly selected human cDNA clones, a data base containing more than 2 million ESTs obtained from over 750 different cDNA libraries has been generated by Human Genome Sciences, Inc. A specific homology and motif search using the known amino acid sequence motif of TNF family members against this data base revealed several ESTs having homology to members of the TNF family. One full-length cDNA clone (HNEDU15) encoding an intact NH_2-terminal signal peptide was isolated from a human neutrophil library and selected for further investigation. The complete cDNA sequence of both strands of this clone was determined, and its homology to TNF was confirmed. This gene product was named THANK. THANK is a 285-amino acid-long type II transmembrane protein. The transmembrane domain was found to be located between amino acid residues 47 through 77 (Fig. 1*A*).

The cDNA encoding the extracellular domain of THANK (amino acids 76–285) was amplified employing the polymerase chain reaction technique using the following primers: 5′ *Bam*HI, GCGGGATCCCAGCCTCCGGGCAGAGC and 3′ *Xba*I, GCGTCTAGATCACAGCACTTTCAATGC. The amplified fragment was purified, digested with *Bam*HI and *Xba*I I, and cloned into a baculovirus expression vector pA2-GP, derived from pVL94. The cloning, expression, and confirmation of the identity of the cloned product were performed using standard techniques (18).

Recombinant THANK was purified from the clarified culture supernatant of 92-h postinfected Sf9 cells. The protein was stepwise purified by cation and anion exchange chromatography. The purified THANK was analyzed for purity by 12% SDS-PAGE and by Western blot analysis.

Northern Blot Analysis—Two multiple human tissue Northern blots containing 2 μg of poly(A)$^+$ RNA per lane of various tissues (CLONTECH) were probed with ^{32}P-labeled THANK cDNA. RNA from a selected panel of human cell lines were probed following the same technique.

Production of THANK Antibodies—Antibodies against THANK were raised by injecting 0.2 mg of purified recombinant antigen in Freund's complete adjuvant (Difco) subcutaneously into a rabbit. After 3 weeks,

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[1] The abbreviations used are: TNF, tumor necrosis factor; NF-κB, nuclear transcription factor-κB; EMSA, electrophoretic mobility shift assay; AP-1, activator protein 1; JNK, NH_2-terminal c-Jun kinase; PARP, poly(ADP-ribose) polymerase; EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis.

the injection was repeated, and the rabbit was bled every 3rd week. The specificity of the antiserum was tested by enzyme-linked immunosorbent assay and Western blot.

Receptor Binding Assay—TNF receptor binding assay was performed following a modified procedure described previously from our laboratory (19). Briefly, 0.5×10^6 cells/well (triplicate well) in 100 μl of binding medium (RPMI 1640 containing 10% fetal bovine serum) were incubated with ^{125}I-labeled TNF (2.5×10^5 cpm/well, specific activity 40 mCi/mg) either alone (total binding) or in the presence of 20 nM unlabeled TNF (nonspecific binding) or 150 nM unlabeled THANK in an ice bath for 1 h. Thereafter, cells were washed three times with ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin to remove unbound ^{125}I-TNF. The cells were dried at 80 °C, and the cell-bound radioactivity was determined in a γ counter (Cobra-Auto Gamma, Packard Instrument Co.)

Electrophoretic Mobility Shift Assay (EMSA)—NF-κB activation was analyzed by EMSA as described previously (20, 21). In brief, 6-μg nuclear extracts prepared from THANK-treated cells were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide for 15 min at 37 °C and the DNA-protein complex resolved in 7.5% native polyacrylamide gel. The specificity of binding was examined by competition with unlabeled 100-fold excess oligonucleotide. The specificity of binding was also determined by supershift of the DNA-protein complex using specific and irrelevant antibodies. The samples of supershift experiments were resolved on 5.5% native gels. The radioactive bands from dried gels were visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Western Blot of THANK—Purified THANK sample was resolved on 12% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and probed with polyclonal antibodies (1:6000) raised in rabbits. The blot was then treated with horseradish peroxidase-conjugated secondary antibodies and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

c-Jun Kinase Assay—The c-Jun kinase assay was performed by a modified method as described earlier (22). Briefly, 100-μg cytoplasmic extracts were treated with anti-JNK1 antibodies, precipitated the immune complexes with protein A/G-Sepharose beads (Pierce), and assayed for the enzymatic activity by using glutathione *S*-transferase-Jun (amino acids 1–79) as substrate (2 μg) in the presence of 10 μCi of [^{32}P]ATP. The kinase reaction was carried out by incubating the above mixture at 30 °C in kinase assay buffer for 15 min. The reaction was stopped by adding SDS sample buffer, followed by boiling. Finally, protein was resolved on a 9% acrylamide gel under reduced conditions. The radioactive bands of the dried gel were visualized and quantitated by PhosphorImager as mentioned previously. To determine the total amount of JNK1 protein, 30 μg of the cytoplasmic extracts were loaded on 9% acrylamide gels. After electrophoresis, the protein was transferred to nitrocellulose membranes, blocked with 5% non-fat milk protein, and probed with rabbit polyclonal antibodies (1:3000) against JNK1. The blots were then reacted with horseradish peroxidase-conjugated secondary antibodies and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Cytotoxicity Assays—The cytotoxic effects of THANK against tumor cells were measured by modified tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay described earlier (23) and by its ability to activate caspase-3 leading to cleavage of poly(ADP-ribose) polymerase (PARP) (24). For cytotoxicity, 5×10^3 cells in 0.1 ml were plated in triplicate in 96-well plates and exposed to variable concentrations of either THANK or TNF (for comparison) in 0.1 ml. After 72-h incubation at 37 °C, cells were examined for viability. To estimate caspase-3 activation by PARP cleavage, cell extracts (50 μg/sample) were resolved on 7.5% acrylamide gels, electrophoresed, transferred to nitrocellulose membranes, blocked with 5% non-fat milk protein, probed with PARP monoclonal antibody (1:3000), and detected by ECL as indicated above.

RESULTS AND DISCUSSION

Identification, Sequence, and Purification of THANK—The predicted amino acid sequence of mature THANK (112–285) is 15, 16, 18, and 19% identical to LIGHT, FasL, TNF, and LT-α, respectively (Fig. 1*A*). The cDNA for this novel cytokine was cloned and expressed in a baculovirus expression system. In CM cellulose cation exchange chromatography, THANK eluted first with 1 M NaCl (fraction A) and then with 1.5 M NaCl (fraction B). Fraction B had an approximate molecular mass of 20 kDa on 12% SDS-PAGE with an amino-terminal sequence

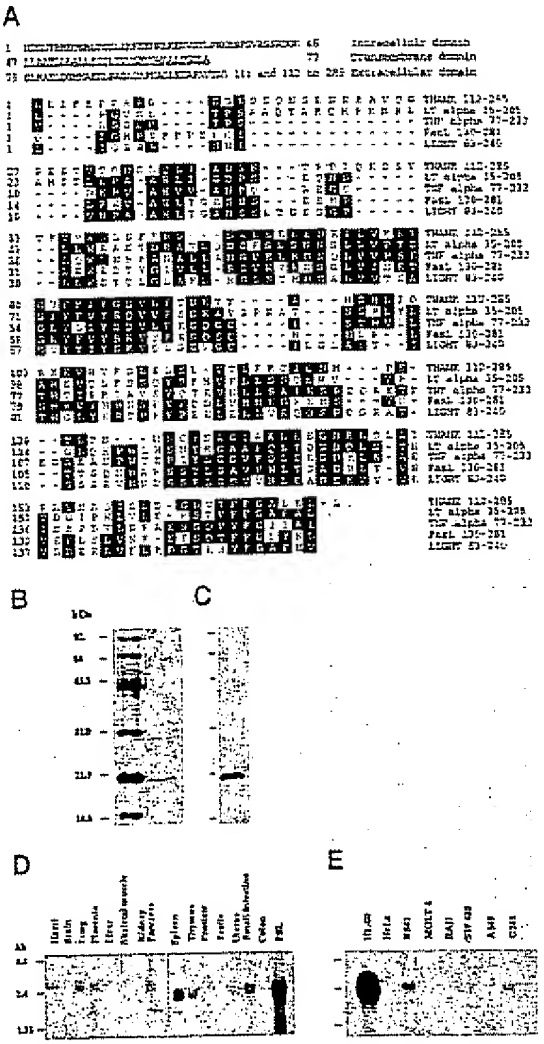

FIG. 1. *A*, amino acid sequence of THANK and its comparison with mature form of TNF, LT, FasL, and LIGHT. The *shaded area* indicates homology with LT, TNF, FasL, and LIGHT. *B*, SDS-PAGE analysis of THANK (fraction B). *C*, Western blot analysis of THANK (fraction B). *D*, tissue distribution of THANK mRNA. *E*, expression of THANK mRNA by various cell lines. *PBL*, peripheral blood leukocytes.

starting at AVQGP, whereas fraction A contained an additional peptide of 3 kDa with an amino-terminal sequence LKIFEPP (Fig. 1*B*). An apparently higher molecular size obtained by SDS-PAGE than that predicted from the number of amino acids suggested a post-translational modification. The amino acid sequence of the mature THANK lacked, however, the potential *N*-glycosylation site. Polyclonal antibodies prepared against THANK recognized the cytokine on Western blot (Fig. 1*C*).

Tissue and Cell Line Distribution of THANK—Northern blot analysis indicated that THANK was expressed in peripheral blood leukocytes, spleen, thymus, lung, placenta, small intestine, and pancreas; with highest expression in peripheral blood leukocytes (Fig. 1*D*). Analysis of the cell line blot (CLONTECH) revealed very high expression in HL60, detectable expression in K562, A549, and G361, and no detectable transcript in HeLa,

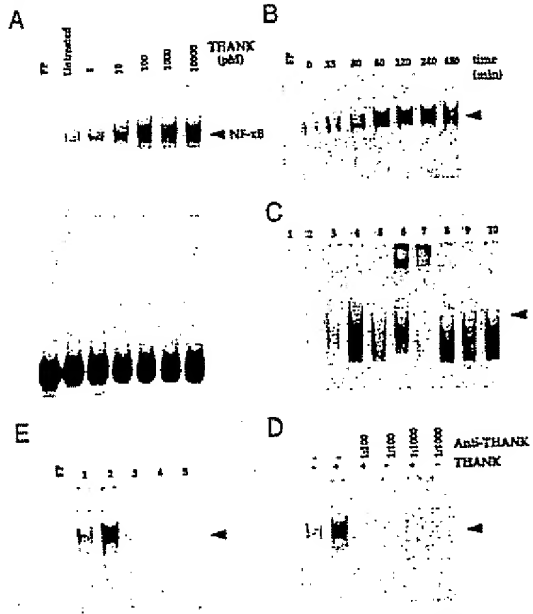

FIG. 2. *A*, dose response of THANK-induced NF-κB activation. U937 cells (2×10^6/ml) were treated with different concentrations of THANK for 60 min at 37 °C and then assayed for NF-κB by EMSA. *B*, kinetics of NF-κB activation. U937 cells (2×10^6/ml) were treated with 1 nM of THANK for various lengths of time. *C*, supershift and specificity of NF-κB. Nuclear extract of THANK-treated cells (*lane 4*) was incubated at room temperature for 60 min with anti-p50 (*lane 5*), anti-p65 (*lane 6*), mixture of anti-p50 and anti-p65 (*lane 7*), anti-c-Rel (*lane 8*), anti-cyclin D1 (*lane 9*), preimmune serum (*lane 10*), unlabeled NF-κB oligonucleotide (*lane 2*) and then assayed for NF-κB. *Lane 1* shows results for free probe, and *lanes 3* and *4* show the THANK-untreated and -treated cells, respectively. *D*, effect of anti-THANK polyclonal antibodies on THANK-induced NF-κB activation in U937 cells. THANK was preincubated with anti-THANK antibodies at a dilution of 1:100 or 1:1000 before cells were exposed. *E*, effect of trypsinization and heat denaturation on the ability of THANK to activate NF-κB in U937 cells. THANK was treated with 0.25% trypsin at 37 °C for 60 min and then checked for its ability to activate NF-κB (*lane 3*). The effect of trypsin alone is shown in *lane 4*. THANK was boiled at 100 °C for 10 min and used for the activation of NF-κB (*lane 5*). *Lanes 1* and *2* represent NF-κB activation for untreated and THANK-treated U937 cells, respectively.

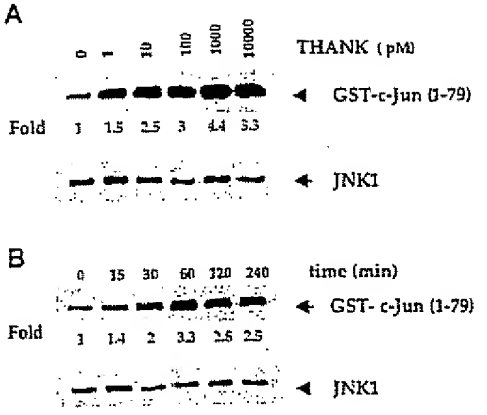

FIG. 3. *A*, dose response of THANK-induced JNK activation. U937 cells (2×10^6/ml) were treated with different concentrations of THANK for 1 h at 37 °C and assayed for JNK activation as described under "Materials and Methods." The *lower panel* shows equal loading of protein. *B*, kinetics of THANK-induced activation of JNK. U937 cells (2×10^6/ml) were treated with 1 nM THANK for the indicated time period and assayed for JNK activation. The *lower panel* shows equal loading of protein. *GST*, glutathione *S*-transferase.

MOLT4, Raji, and SW480 cell lines. Thus cells and tissues of the immune system expressed THANK transcripts.

THANK Activates NF-κB—One of the earliest events induced by most members of the TNF superfamily is NF-κB activation (25). The results depicted in Fig. 2, *A* and *B*, indicate that THANK activated NF-κB in a dose- and time-dependent manner. Less than 10 pM THANK was enough to activate NF-κB in U937 cells, although peak activation was obtained at 1 nM. THANK induced optimum NF-κB activation within 60 min at 1 nM; and there was no significant increase thereafter (Fig. 2*B*). The gel shift band was specific, as its formation could be eliminated with excess unlabeled oligonucleotide. It was supershifted by anti-p50 and anti-p65 antibodies only (Fig. 2*C*), thus indicating that the nuclear factor was composed of p50 and p65 subunits. No significant difference was found in the ability to activate NF-κB between the 20- and 23-kDa forms of THANK, indicating that residues 112 through 134 are optional for the biological activity (data not shown).

To ascertain that the observed activation was due to THANK and not a contaminant, the protein was preincubated with anti-THANK polyclonal antibodies before treatment with the cells. Fig. 2*D* shows a lack of NF-κB activation after treatment of THANK with antibodies even at a 1 to 1000 dilution. Antibody against THANK by itself had no effect. To further ascertain that NF-κB activation was due to the proteinaceous nature of THANK, the protein was either digested with trypsin or heat-denatured prior to treatment. Both treatments completely abolished NF-κB activation in U937 cells, confirming that THANK was responsible for this activation (Fig. 2*E*). Although THANK was as potent as TNF with respect to both dose and time required for NF-κB activation, the overall amplitude of response was less with THANK. In this respect the activity of THANK was comparable with LT-α (21).

THANK Activates c-Jun NH_2-terminal Kinase—The activation of JNK is another early event that is initiated by different members of the TNF family (17, 22). THANK activated JNK activity in a time- and dose-dependent manner (Fig. 3, *A* and *B*). At 10 pM the activity increased by 2.5-fold and at 1 nM it reached 4.4-fold. An additional increase in THANK concentration slightly decreased activation (Fig. 3*A*). The peak activation of JNK was observed at 60 min (3.3-fold increase), which gradually decreased thereafter (Fig. 3*B*). These results suggest that, like TNF, THANK transiently activates JNK in U937 cells. The activation of JNK by THANK was not due to an increase in JNK protein levels, as immunoblot analysis demonstrated comparable JNK1 expression at all dose and time points (Fig. 3, *A* and B, *lower panels*).

THANK-induced Cytotoxicity and Caspase-3 Activation—Activations of NF-κB and JNK are early cellular responses to TNF, which are followed by cytotoxic effects to tumor cells. The effect of different concentrations of THANK on the cytotoxic effects against tumor cell lines was examined and compared with that of TNF. Results in Fig. 4*A* show that THANK inhibited the growth of human histiocytic lymphoma U937 cells in a dose-dependent manner. Besides U937, THANK also inhibited the growth of prostate cancer (PC-3), colon cancer (HT-29), cervical carcinoma (HeLa), breast carcinoma (MCF-7), and embryonic kidney cells (A293) (data not shown). The growth inhibition curve of THANK was superimposable with that of TNF, indicating comparable potency.

Degradation of PARP by caspase-3 is one of the hallmarks of apoptosis in tumor cells (26). We found that treatment of U937 cells with THANK for 2 h induced partial cleavage of PARP in

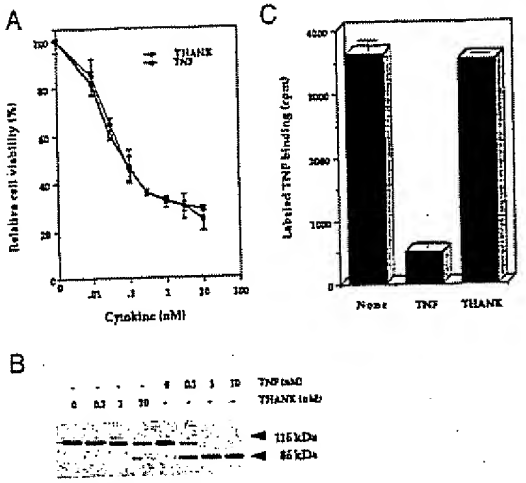

FIG. 4. *A*, dose-dependent cytotoxic effects of THANK against U937 cells. 5×10^3 cells/well were incubated in triplicate with various concentrations of THANK or TNF and then examined for cell viability after 72 h. Untreated control is expressed as 100%. *B*, THANK-induced cleavage of PARP in U937 cells. U937 cells (2×10^6 cells/ml) were treated with 0.1, 1, and 10 nM THANK in the presence of cycloheximide (10 μg/ml) for 2 h at 37 °C. In order to compare the cleavage, TNF was used as a positive control. *C*, competitive inhibition of labeled TNF binding to U937 cells by unlabeled TNF (20 nM) and THANK (150 nM). U937 cells (0.5×10^6 cells/well) were incubated with 0.25×10^6 cpm of ^{125}I-TNF in an ice bath for 1 h in the presence or absence of the unlabeled competitors. Cell-bound radioactivity was measured in a γ counter. Results are expressed as mean ± S.D.

U937 cells, whereas TNF almost completely cleaved PARP under these conditions (Fig. 4*B*). This suggests that THANK can activate caspase-3, although not so strongly as TNF.

THANK Binds to Receptors Distinct from TNF Receptors—Previously we have shown that TNF and LT, which share homology with each other to the same extent as THANK, bind to the same cell surface receptors (4). Since THANK has significant amino acid sequence homology with TNF, and like TNF exhibits cytotoxic effects, and activates NF-κB and JNK, we examined its binding to the TNF receptor. The receptor binding results (Fig. 4*C*) show that 20 nM unlabeled TNF almost completely blocked the binding of ^{125}I-labeled TNF to U937 cells, whereas 150 nM unlabeled THANK did not compete for ^{125}I-TNF binding sites. These results suggest that THANK interacts with U937 cells through a receptor distinct from that for TNF.

In summary, we describe here the identification of a novel cytokine expressed by hematopoietic cells that can, like TNF and LT-α, activate NF-κB and JNK and inhibit the growth of a wide variety of tumor cells. Although the structure of THANK also exhibits homology to FasL and LIGHT, the latter have not been reported to activate NF-κB. Our preliminary results by using flow cytometry indicate that THANK protein is expressed by promyelomonocytic HL-60 cells (data not shown). Because THANK is expressed by hematopoietic cells, it appears to be similar to LT-α and dissimilar from other members of the TNF superfamily. Among all the members of the TNF superfamily, THANK exhibits cytotoxic effects similar to TNF and LT-α. Whether THANK exhibits immunomodulatory activities and *in vivo* antitumor activities is currently under investigation.

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